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Enhanced contrast of bacteriophage plaques in *Salmonella* with ferric ammonium citrate and sodium thiosulfate (FACST) and tetrazolium red (TZR) ☆

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Abstract

Visualization of bacteriophage plaques may be enhanced by addition of ferric ammonium citrate and sodium thiosulfate (FACST) or 2,3,5-triphenyltetrazolium chloride (tetrazolium red, TZR) to the soft agar layer of a traditional bacteriophage plaque assay. Background color from these reagents improved contrast between clear plaques and turbid host lawns in trypticase soy agar (TSA) plates. Enhancement by FACST is based on reaction with hydrogen sulfide gas (H_2S) produced by some strains of bacteria and was tested here using H_2S+ and H_2S− strains of *Salmonella enterica* subsp. *enterica* with a bacteriophage (*Podoviridae*) isolated from swine lagoon effluent. Only the H_2S+ strain produced dark brown-black color in FACST-amended agar. Both strains showed bright pinkish-red color in TZR-amended agar. Color intensity for both reagents decreased with decreasing concentrations of the reagents. Contrast in FACST-amended plates appeared greater than that with TZR, but diminished after 12 h, while contrast in TZR-amended plates remained constant. At the concentrations tested, neither reagent affected plaque counts in the H2S+ strain. The FACST should be useful in bacteriophage plaque assays with H_2S+ strains of *Salmonella* and other H_2S+ bacteria. Published by Elsevier B.V.

Keywords: Bacteriophage; Plaque assay; *Salmonella*; Tetrazolium; Thiosulfate; H_2S

1. Introduction

Dyes have been added to bacterial culture media for the identification, characterization, and enumeration of bacteria and bacteriophages for decades (Mac Faddin, 2000). Levine (1957) incorporated dyes in indicator agar used to distinguish plaque-morphology mutants of bacteriophage P22. Tetrazolium dyes have been widely used (Berman et al., 1992; Fraser and Crum, 1975; Sobsey et al., 1990). Effects of tetrazolium dyes on bacteriophage plaque assay titers have been studied for several bacteriophage families (Hurst et al., 1994). Tetrazolium red (TZR; 2,3,5-triphenyltetrazolium chloride) was shown to suppress replication of P22, a member of the *Podoviridae*, at a concentration of 300 μg/ml, but not at 150 or 50 μg/ml (Hurst et al., 1994).

Recently, our studies of *Salmonella* bacteriophages associated with swine effluent lagoons produced several bacteriophages with *Podoviridae* morphology (McLaughlin et al., 2003). This recent study utilized

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traditional agar layer plaque assays as described by Adams (1950, 1959). The clear phage plaques in the cream-color bacterial lawns of this assay were not readily distinguished without dark field lighting. A simple method of enhancing the contrast between plaques and host background was needed to make visual plaque counts easier in small-scale experiments with relatively few plates and to facilitate future digital automation in larger scale experiments with relatively larger numbers of plates.

The initial objectives of the present study were: 1) to determine the utility of TZR in plaque assays to enhance contrast in tests of the *Podoviridae* phage from swine effluent; and 2) to determine whether lower concentrations of TZR might be as effective in enhancing visibility of plaques, thus further reducing the possibility of dye interference with bacteriophage replication. Because the bacterial host for the phage is a *Salmonella*, we also tested the use of ferric ammonium citrate (FAC) and sodium thiosulfate (ST), which we routinely used to aid in the detection of *Salmonella*. In well-known reactions, sulfate-reducing strains of *Salmonella* break down thiosulfate to sulfite and H_2S gas. The presence of H_2S is detected by its reaction with ferric ions from the heavy iron salt, ferric ammonium citrate, to produce a brown-black precipitate, which marks *Salmonella* colonies in clear TSA plates. The FACST was included in the current study with the objective of testing its use for marking bacterial host lawns and enhancing plaque visibility in H_2S+ *Salmonella*. Its effects on bacteriophage plaque counts, and stability of the enhancing color reaction were also assessed.

2. Materials and methods

2.1. Cultures

Salmonella enterica subsp. *enterica* (ex Kauffmann and Edwards) Le Minor and Popoff serovar Enteritidis (ATCC 13076, referred to here as R05), *S. enterica* subsp. *enterica* (ex Kauffmann and Edwards) Le Minor and Popoff serovar Typhimurium (ATCC 700730, referred to here as R37) and bacteriophage isolate PR05-43 (*Podoviridae*) were used in this trial. PR05-43 was isolated from swine lagoon effluent by single plaque selection on *Salmonella* R05 as described (McLaughlin et al., 2003) and subsequently propagated in *Salmonella* R05.

2.2. Culture media

Salmonella R05 and R37 cultures were grown in trypticase soy broth (TSB) and trypticase soy agar (TSA) (BBL, Becton Dickinson, Cockeysville, MD, USA). Bacteriophage PR05-43 was isolated and cultured using *Salmonella* R05 in TSA and TSB.

2.3. H_2S production by Salmonella isolates

Production of H_2S by *Salmonella* strains R05 and R37 in the presence of FACST was determined along with 14 other *Salmonella* isolates using a 48-pin replicator to transfer 5 μl droplets of TSB cultures from a 96-well microtiter plate onto the surface of soft TSA containing FACST (0.8 g FAC and 6.8 g ST per liter). The H_2S reactions of the *Salmonella* isolates were also determined using API 20 E test strips according to the manufacturer's recommendations (bioMérieux Vitek, Inc., Hazelwood, Missouri).

2.4. Preparation of phage lysate

A turbid overnight standing culture of *Salmonella* R05 in TSB was inoculated with bacteriophage PR05-43, and incubated at 35 °C, 5.5 h until the turbidity cleared. Chloroform was added to 2.5% (*v*/*v*) and mixed thoroughly to complete bacterial cell lysis and the lysate+chloroform mixture was held at room temperature 30 min. Lysate was clarified by centrifugation at 12,400 ×*g* for 10 min at 5 °C. Clarified lysate was passed through a 0.45μ filter and stored in an amber vial at 5 °C. Phage titer of the clarified and filtered lysate was determined in an agar layer plaque assay, as described below. The titer of the PR05-43 bacteriophage lysate stock used in this study was estimated as 1.75×10^7 pfu/ml. Dilutions from this stock were made in suspension media (SM: 0.01 M $MgSO_4$; 0.1 M NaCl; 0.05 M Tris, pH 7.5; 0.01% gelatin).

2.5. Agar layer plaque assays

Bacteriophage lysate titer was determined by plating 100 μl from 10-fold dilutions of phage lysate in SM with 100 μl of fresh log-phase *Salmonella* R05 in TSB, and 5.0 ml of soft TSA (0.75% agar melted and tempered to 45 °C). Test dilutions were mixed by vortexing and dispensed uniformly over the surface of 20 ml of hard TSA in 96-mm-diameter plates. Soft agar overlays were allowed to harden at room temperature then plates were inverted and incubated overnight at 35 °C. Plaque forming units (pfu) were counted for each plate and the bacteriophage titer of the lysate was calculated.

Tests of FACST and TZR were similarly done, using 100 μl of bacteriophage lysate diluted in SM,

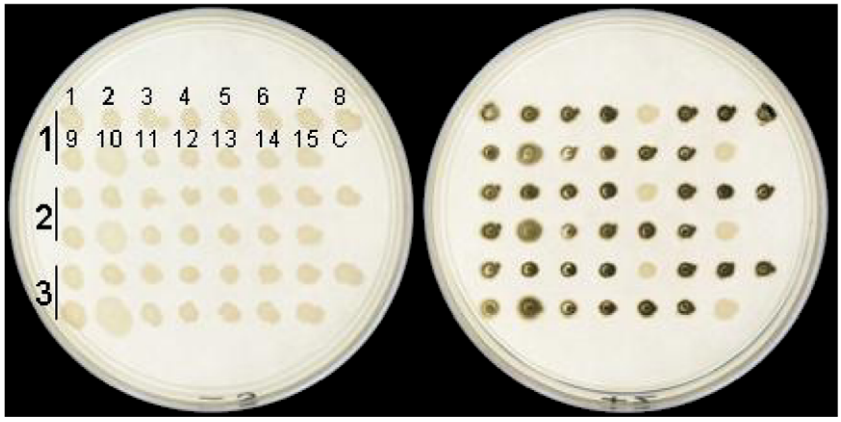

Fig. 1. *Salmonella* isolates spotted on the surface of soft TSA with (right) and without (left) FACST. Isolates placed in consecutively numbered positions 1–15 are identified in Table 1. Each numbered isolate and a sterile TSB control (C) were replicated three times in the same order and orientation on each plate. Note that reference isolates in positions number 5 and 15 were H_2S-. Isolates R05 (H_2S+) and R37 (H_2S-), which were used in other tests in the present study, are in positions 2 and 5, respectively.

100 μl of *Salmonella* culture (either R05 or R37) in TSB, and 5.0 ml of soft TSA (± FACST or TZR), which was mixed and distributed evenly over 20 ml TSA in 96-mm-diam plates.

Initial tests of FACST were made by adding FAC and ST to the soft TSA to the concentrations described above for culture media (1 × FACST). Subsequent tests were made by adding a filter-sterilized concentrated stock solution of FACST to the soft agar to final FACST levels of 1×, 0.5× and 0.25× in the soft TSA layer. Initial tests of TZR were conducted by adding TZR to a final level of 189 μg/ml in the soft TSA. Subsequent tests of TZR were done by adding TZR from a filter-sterilized concentrated stock to the soft TSA to final concentrations of 50, 25, and 12.5 μg/ml. Tests to compare effects of the three concentrations each of FACST and TZR versus a TSA control were conducted using 8 replicate plates for each test combination. Plaques were counted after 12 h and plate counts (pfu/plate) were converted to log units and analyzed by Tukey's test.

3. Results and discussion

The H_2S reactions of *Salmonella* isolates were consistent in spot test cultures on TSA+FACST

Table 1
Identity and H_2S production of *Salmonella* isolates

Placement position no. in Fig. 1	Reference no. for this study	ATCC no.	Serovar	H_2S produced FACST[a]	H_2S produced API 20 E[b]
1	R04	14028	*Typhimurium*	+	+
2	R05	13076	*Enteritidis*	+	+
3	R21	43971	*Typhimurium*	+	+
4	R36	13311	*Typhimurium*	+	+
5	R37	700730	*Typhimurium*	−	−
6	R38	BAA-189	*Typhimurium*	+	+
7	R39	BAA-190	*Typhimurium*	+	+
8	R40	BAA-191	*Typhimurium*	+	+
9	R41	BAA-215	*Typhimurium*	+	+
10	R42	BAA-707	Agona	+	+
11	R43	BAA-708	*Enteritidis*	+	+
12	R44	BAA-709	Michigan	+	+
13	R45	BAA-710	Montevideo	+	+
14	R46	BAA-711	Gaminara	+	+
15	R47	BAA-712	*Typhimurium*	−	−
C	TSB	Control		No growth	−

[a] Reaction with ferric ammonim citrate and sodium thiosulfate.
[b] Reaction with bioMerieux Vitek, Inc. test kit for *Enterobacteriaceae*.

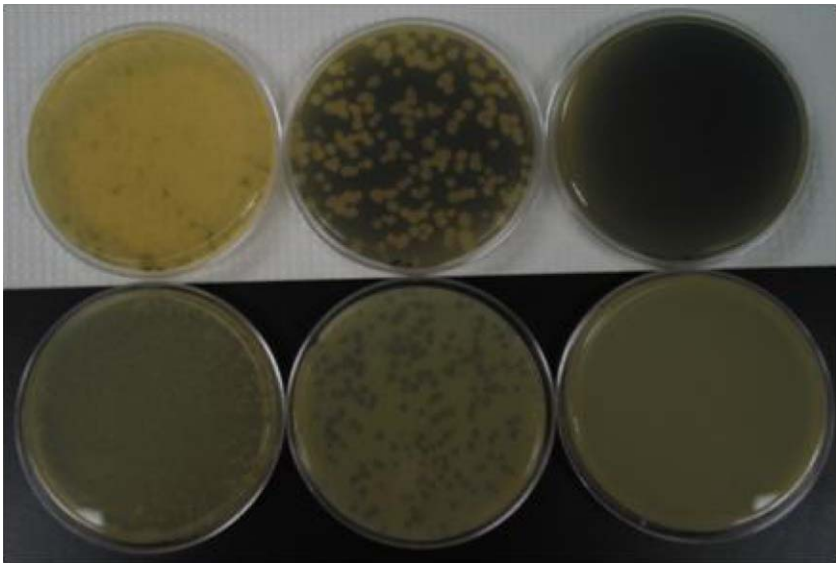

Fig. 2. Agar layer plaque assays with bacteriophage PR05-43 on *Salmonella* R05 in soft TSA with (top, against white background) and without (bottom, against black background) FACST. All plates were inoculated with the same concentration of *Salmonella*. Plates on the left were inoculated with a high concentration of bacteriophage, which produced confluent plaques too numerous to count, resulting in almost complete absence of black color in the FACST plate (top left). Plates in the middle were inoculated with a 10-fold dilution of bacteriophage compared to that used on the left. Plates on the right were inoculated with a sterile SM control without bacteriophage.

(Fig. 1) and API 20 E test strips (Table 1). These test results confirmed that *Salmonella* strain R37 was H_2S- and that strain R05 was H_2S+ and suitable for use in comparing TSA+FACST, TSA+TZR, and TSA.

Initial tests of FACST showed marked contrast between clear plaques and bacterial host lawns of the H_2S+ strain which developed dark brown–black color (Fig. 2). Contrast enhancement between the host lawn with FACST and the clear plaques was only observed

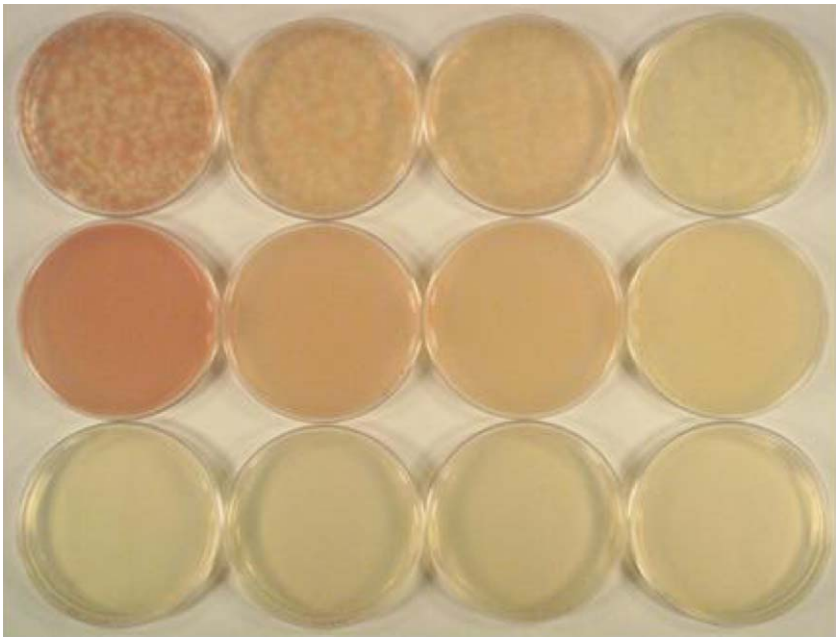

Fig. 3. Agar layer plaque assays with 50 (left column), 25 (column second from left), 12.5 (column third from left) and 0 μg/ml TZR (right column). Plates in the top and middle rows contain *Salmonella* R05. Plates in the top and bottom rows contain bacteriophage PR05-43.

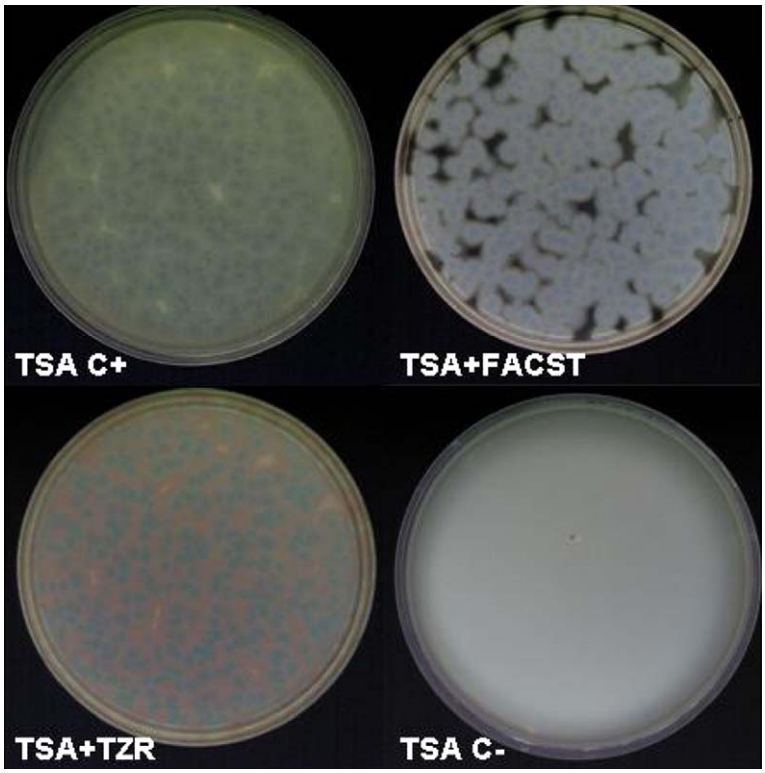

Fig. 4. Color contrast of back and indirectly lighted agar layer plaque assays on *Salmonella* R05 in soft TSA. TSA controls with (C+) and without (C−) bacteriophage PR05-43. Plaques on TSA with TZR (+TZR) and with FACST (+FACST).

with the H_2S+ strain. Subsequent tests of FACST with the H_2S− strain showed no color development and bacterial host lawns that were indistinguishable from those of the TSA control without FACST. The black color imparted in H_2S+ lawns, however, began to fade rapidly after about 12 h and eventually cleared completely. The precipitate forming reaction of ferric ions with H_2S is dependent upon an acid pH and carbohydrates as a source of H^+ ions. If oxidative exhaustion of carbohydrate occurs, ammonia is produced, pH increases, the black precipitate dissolves and the color clears. Therefore, for optimum contrast using FACST, plaque counts should be made within 12 h of plating.

Table 2
Tukey's test analysis of log transformed plate counts (pfu/plate) of agar layer plaque assays comparing FACST, TZR and TSA

Treatment	Count	Mean	Std. dev.	Std. err.	Diff from TSA
TSA	8	5.8226	0.0270	0.0095	0
FACST 1×	8	5.7646	0.0354	0.0125	−0.0580
FACST .5×	8	5.8387	0.0187	0.0066	0.0161
FACST .25×	8	5.8028	0.0185	0.0066	−0.0198
TZR 50 μg/ml	8	5.8947	0.0307	0.0109	0.0721
TZR 25 μg/ml	8	5.8080	0.0269	0.0095	−0.0146
TZR 12.5 μg/ml	8	5.8502	0.0162	0.0057	0.0276
Tukey's test value		0.014676			

Tests of TZR showed bright pinkish-red color in bacterial host lawns (Fig. 3), with diminishing intensity correlated with lower concentrations of TZR in the soft TSA. Clear plaques contrasted well with the red bacterial lawn in plates containing TZR at 50 μg/ml. Plaque and lawn formation with FACST, TZR, and TSA are compared using indirectly back lighted plates in Fig. 4.

Paired comparisons of FACST and TZR treatments with the TSA control showed that all differed statistically from the control except TZR at 25 μg/ml, however, examination of the data showed low standard deviations and relatively small differences between the respective means, with a range from 0.0146 to 0.0721 log units and, therefore, practically no biological significance (Table 2).

4. Conclusion

Addition of FACST or TZR to soft TSA overlays in agar layer plaque assays is a simple and reliable method to enhance plaque discrimination and enumeration of bacteriophage PR05-43 in H_2S+ *Salmonella*

R05 lawns. The additive concentrations and test conditions used here had no significant biological effect on plaque counts. Enhancement of plaque assays by addition of TZR has previously been shown to be an effective tool which could be applied to a broad range of bacterial families (Hurst et al., 1994). Based on that earlier work and results presented here, TZR enhancement should be applicable to all bacteriophage and *Salmonella* host combinations. Enhancement by FACST, however, is new and uniquely applicable to use with H_2S+ bacterial strains. The chief disadvantage of FACST enhancement is the rapid diminution of color after 12–14 h.

Todar (2005) listed 'H_2S production from thiosulfate' among characteristics shared by most strains of *Salmonella* subgroup I (*S. enterica* subsp. *enterica*). Product literature with the API 20E (bioMérieux Vitek) test kit for identification of Enterobacteriaceae listed 64% of *S. enterica* subsp. *enterica* and 99% of *S. enterica* subsp. *arizonae* as H_2S+. FACST enhancement of plaque assays should, therefore, be applicable to most strains of these subgroups. It is expected that FACST enhancement of plaque assays would also be applicable to H_2S+ strains of other members of the Enterobacteriaceae, including *Citrobacter*, *Edwardsiella* and *Proteus*.

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